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Isolation of the Novel Agent from Human Stool Samples That Is Associated with Sporadic Non-A, Non-B Hepatitis

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The agent(s) responsible for sporadic non-A, non-B hepatitis in humans was serially transmitted in rhesus monkeys by intravenous inoculation of the stool extract from a patient. A novel agent called HFV (hepatitis French [origin] virus) was present as 27- to 37-nm particles in the infectious stool extract. Hepatopathic lesions were noticed in infected monkeys during the acute phase of illness. The purified viral 27- to 37-nm particles consist of a double-stranded DNA of ~20 kb and are detected in infected monkey liver. Analysis of cell culture detects the ~20-kb-long viral DNA in stool samples from infected monkeys and sporadic enteric non-A, non-B hepatitis patients. Furthermore, the 27- to 37-nm viral particles were able to protect monkeys challenged with infectious stool extract. Our results indicate that 27- to 37-nm virus like particles are responsible for sporadic non-A, non-B hepatitis in rhesus monkeys.

Viral hepatitis may be caused by hepatitis virus types A, B, C, D, and E and an unknown number of not yet identified non-A, non-B hepatitis viruses.

Hepatitis E virus (HEV) is the causative agent of approximately 50 to 100% cases of enterically transmitted sporadic and epidemic hepatitis (2, 6, 9). HEV is 27 to 34 nm in size and consists of a positive-sense RNA 7.5 kb in length (1, 2).

Besides the epidemics of enterically transmitted HEV hepatitis, isolated cases of nonparenterally acquired non-A, non-B hepatitis have been reported. Such cases, referred to as sporadic, were from northwest England (1), northern Italy (3), France (7, 10), the United States (4), and India (5). A 5-kb-long DNA virus was reported to be the causative agent of sporadic non-A, non-B hepatitis among German patients (11). Its sequence does not resemble any of the known sequences present in the gene data bank.

Recently we described a primate model for sporadic non-A, non-B hepatitis (10). Indian rhesus monkeys (*Macaca mulatta*) inoculated with stool extracts from sporadic non-A, non-B hepatitis patients developed hepatitis, and the infectious agent was excreted in the stools during the preicteric phase. Both large (50- to 100-nm) and small (27- to 37-nm) viruslike particles (VLPs), both or either of which could be the infectious agent, were observed in the stools. This report demonstrates that the small particles are infectious in monkeys, conferring immunity against the disease, and that the genetic material of the virus is a double-stranded DNA ~20 kb in size.

MATERIALS AND METHODS

Nonhuman primates. Wild-caught rhesus monkeys (*M. mulatta*) from northern India were used. The monkeys were quarantined for 90 days, during which time they were tested with tuberculin (old tuberculin obtained from the Institute for Sera Bacteriological Production and Research, Budapest, Hungary) every 2 weeks. Monkeys negative in this test over the quarantine period were taken for the study. Monkeys were bled before inoculation to measure the basal levels of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and bilirubin. SGOT, SGPT, and bilirubin estimations were done with commercial kits supplied by M/s Northern Biologicals and Chemicals Pvt. Ltd., Noida, India.

Stool samples. Stool specimens were collected from five well-defined French sporadic non-A, non-B hepatitis patients (9a, 10). These patients were suffering from jaundice with high levels of SGOT, and they were all negative for hepatitis A and B viruses and Epstein-Barr virus. Stool specimens were collected after the first week after the appearance of jaundice. Stool specimens were homogenized as 10% (wt/vol) suspensions in 0.01 M phosphate-buffered saline (PBS) (pH 7.4). The homogenates were clarified by centrifugation (10,000 × *g* for 20 min) and filtered through a 0.22-μm-pore-size filter (Millipore). The presence of the antigen was confirmed by enzyme-linked immunosorbent assay (ELISA) as described below. The extracts were kept in a sterile condition at 4°C.

ELISA for the detection of HFV (hepatitis French [origin] virus) antigen. For purification of immunoglobulin M (IgM), Sephadex G-200 was used. Three major peaks were obtained; peak I contained IgM and X macroglobulin plus some lipoproteins. After concentration of peak fractions, IgM was confirmed by immunoelectrophoresis and reactivity with class-specific sera. For purification of IgG, DEAE-cellulose (DE52; Whatman) was equilibrated under conditions of pH and ionic strength which allow all serum proteins except IgG to bind. For this purpose, 5 mM Tris-phosphate buffer (pH 8.0) was used. The serum was dialyzed and equilibrated with this buffer. Sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis and immunoelectrophoresis were done with anti-whole monkey serum to identify IgG and other impurities. Anti-IgG was used to confirm the presence of IgG in the fractions concentrated.

Ninety-six well flat-bottom Nunc ELISA plates were coated with 100 μl of IgM anti-HFV antibody (25 μg/ml) for 1 h at 37°C and overnight at 4°C. (IgM and IgG anti-HFV antibodies were isolated from a rhesus monkey infected with HFV). Plates were washed four times with washing buffer containing 10 mM PBS and 0.1% Tween 20. Vacant sites were saturated with 1% bovine serum albumin (BSA) and 0.1% Tween 20 in 10 mM PBS for 2 h at 37°C. After saturation, the rest was discarded and stool inocula with appropriate controls were incubated for 1 h at 37°C and overnight at 4°C. The next day,

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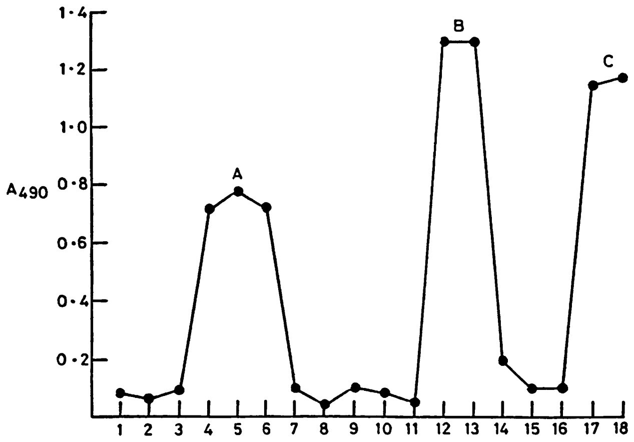

FIG. 1. Distribution of HFV Ag activity in different sucrose gradient fractions after rate zonal centrifugation. HFV Ag-positive stool extracts containing both large and small VLPs were size fractionated on a 10 to 60% sucrose gradient. Fractions 1 to 18 were collected from the bottom of the tube and tested by HFV Ag ELISA after a 20-fold dilution in PBS.

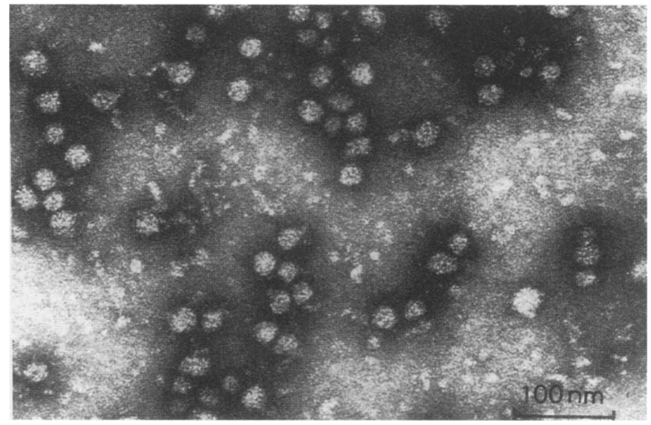

FIG. 2. Electron micrograph of negatively stained 27- to 37-nm VLPs purified from monkey stool.

after four washes with washing buffer, 100 μl of revealing antibody (IgG anti-HFV conjugated to horseradish peroxidase; 5 μg/ml) in 1% BSA–0.1% Tween 20 containing PBS was added. After incubation for 1 h at 37°C, 100 μl of substrate containing 3 mg of *o*-phenylenediamine per liter and 1 μl of hydrogen peroxide per ml in 100 mM citrate phosphate (pH 5.5) was added in the dark. The reaction was stopped with 1 N H_2SO_4. The optical density at 490 nm was measured.

Viral inoculum. Stool extracts from experimentally infected rhesus monkeys (10% [wt/vol] in 10 mM PBS [pH 7.6]) were homogenized and centrifuged at 10,000 rpm for 15 min. The supernatant was filtered through 0.22-μm-pore-size Millipore filters and tested for HFV antigen (HFV Ag) by ELISA. The ELISA-positive supernatant was centrifuged in an SW41 rotor at 150,000 × *g* for 3 h in a Beckman L8-70M centrifuge. The virus pellet was suspended in approximately 1 ml of GTNE buffer (200 mM glycine, 50 mM Tris, 100 mM NaCl, 1 mM EDTA [pH 7.5]). A discontinuous sucrose gradient was prepared by using defined volumes of particular percentages of sucrose, i.e., 1 ml of 10%, 1.5 ml of 20%, 2 ml of 30%, 2 ml of 40%, 1.5 ml of 50%, and 1 ml of 60%, in 10 mM Tris-HCl (pH 7.6). This gradient was made in a 10.5-ml ultracentrifuge tube with an SW41 rotor. The viral preparation was layered onto this gradient, and weight equilibration of the tubes was done. Ultracentrifugation was carried out for 6 h at 150,000 × *g*. At the end of the run, 0.6-ml fractions were collected from each tube by bottom puncture, using a Beckman fraction recovery system.

Each fraction was diluted 25-fold in PBS and tested by ELISA for the presence of antigen. All fractions were analyzed by ELISA. Positive fractions were recentrifuged on a 10 to 60% sucrose gradient separately under similar conditions and tested by ELISA for antigen positivity. ELISA-positive as well as ELISA-negative fractions were subjected to transmission electron microscopy to check for the presence of particles.

Liver biopsies. Wedge biopsies were taken from rhesus monkeys under ketamine hydrochloride (Themis Chemicals Ltd., Bombay, India) anesthesia. Biopsy specimens were washed in PBS (pH 7.0) and fixed in neutral buffered formalin. They were dehydrated in ascending grades of propanol, cleared in xylene, and embedded in paraffin wax. Five-micrometer sections were stained with hematoxylin and eosin by Lendrum's method (5) for microscopic examination.

Ultrastructural studies. Electron micrographs of virus particles were taken by the negative staining method. Copper grids coated with Formvar and carbon were inverted on a drop of the test sample. The excess of sample was drained off, and the grids were stained with 1% phosphotungstic acid for 3 min. After draining of the excess stain, the grids were air dried and examined under Philips CM10 transmission electron microscope at an accelerating voltage of 100 kV.

DNA isolation and probing of viral DNA from stool and liver. Stool extracts containing 27- to 37-nm particles from the discontinuous sucrose gradient were dialyzed against PBS solution. After dialysis, the viral solution was treated with 0.5 mM EDTA, 0.5% SDS, and 50 μg of proteinase K per ml at 56°C for 1 h, extracted twice with phenol and once with a 1:1 volume of phenol and chloroform, and precipitated in the presence of 0.3 M sodium acetate and ethanol.

Infected monkey liver tissues were washed five times with 0.01 M Tris phosphate buffer (pH 7.6), and then the liver extract was prepared in 0.01 M PBS (pH 7.6). The extract was centrifuged three times at 5,000 × *g*, and the pellet was suspended in 5 volumes of 0.01 M Tris NaCl buffer (pH 8.0). The suspension was sonicated thrice for 2 min in the presence of 0.005 mg of phenylmethylsulfonyl fluoride per ml and then centrifuged at 15,000 × *g* for 20 min. The pellet was washed with Tris phosphate buffer, and the supernatant was layered onto a 20 to 50% sucrose cushion at 38,000 rpm for 6 h. The middle layer was collected and tested by ELISA using HFV antibody. A further sucrose gradient (20 to 60%) was done from the pooled fraction at 38,000 rpm for 6 h. The ELISA-positive fractions were used for the preparation of viral DNA.

Liver and stool DNA samples were subjected to agarose gel electrophoresis by the standard protocol (12). Stool DNA samples were labeled with [^{32}P]dCTP by the random primer method (random priming kit; New England Biolabs, Beverly, Mass.). The resolved bands in agarose were transferred to a GeneScreen Plus (Dupont) nylon membrane. The filter was prehybridized in 5× Denhardt's solution and probed in the same solution overnight. The filter was then washed with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS solution and exposed for 2 days.

Cell culture and virus isolation. The peak B fraction of monkey stool (Fig. 1) containing infectious HFV was used for passaging in a human larynx carcinoma (HEp-2) cell line.

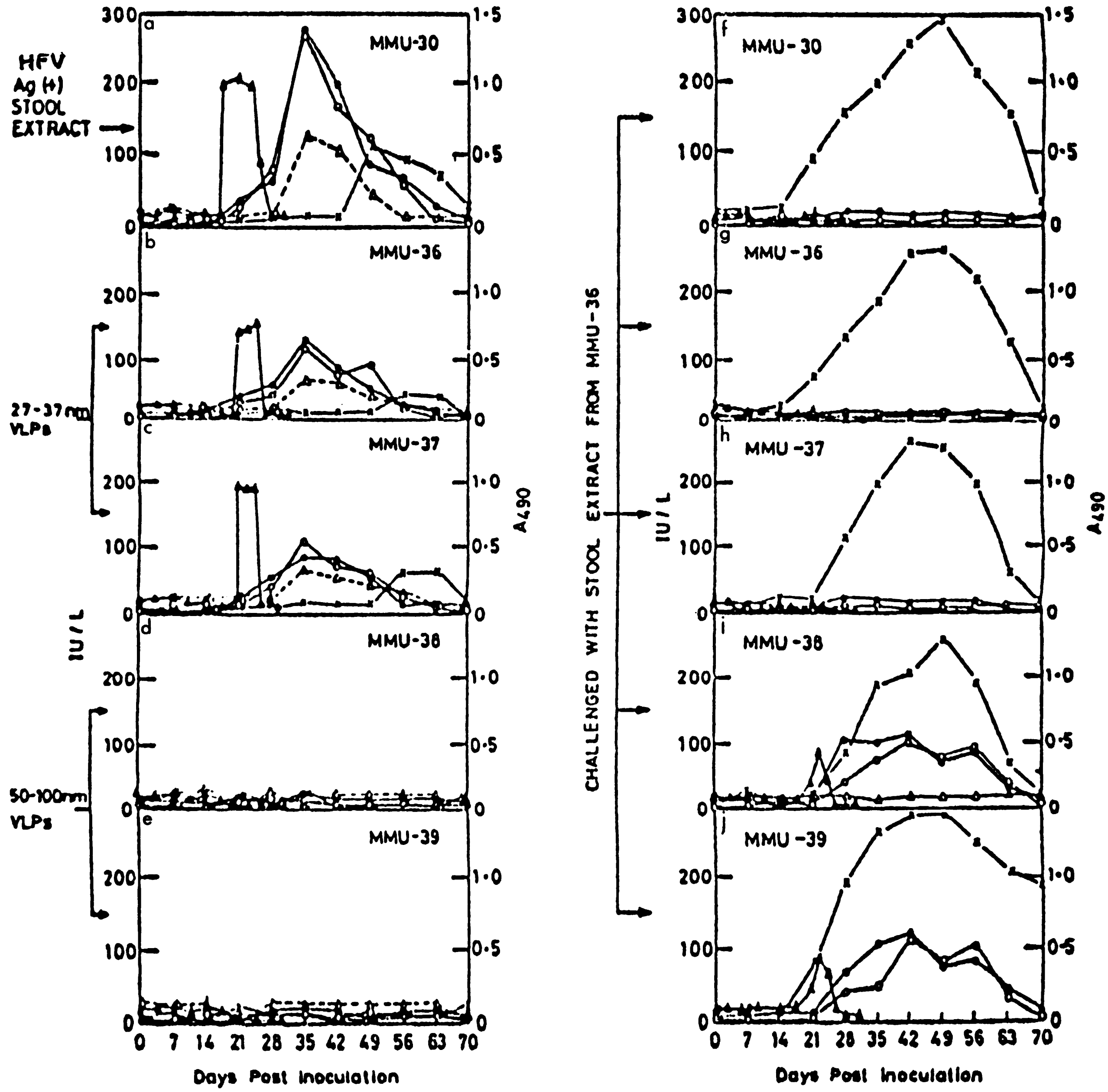

FIG. 3. Serum aminotransferases and HFV Ag in stools of experimentally infected rhesus monkeys. Rhesus monkeys were inoculated with stool extract containing both the large and small VLPs (a), purified 27- to 37-nm VLPs (b and c), and 50- to 100-nm VLPs (d and e). Panels f through j show results from the same monkeys challenged 90 days after the first inoculation with the infectious-phase stool extract of MMU-36. Symbols: O, SGPT; ●, SGOT; ▲, HFV Ag; △, IgM anti-HFV; ×, IgG anti-HFV.

Details of the cell culture and infection by the virus will be described elsewhere (3a). Briefly, 100 μl of HFV (peak B fraction from the sucrose gradient in Fig. 1) was inoculated in a 25-cm^2 flask containing the monolayer of HEp-2 cells and adsorbed for 1 h at 37°C. The inoculum was then decanted and washed with PBS, and the monolayer was replenished with Dulbecco's minimal essential medium with 2% fetal calf serum. Cytopathic effect with rounding of cells was observed after 24 h of incubation and progressed to a maximum at 48 h. The virus was harvested at 48 h and further passaged, and similar cytopathic effect was observed.

Viral DNA was isolated from the infected cell culture supernatant as described above for DNA isolation and probing of viral DNA from stool and liver.

RESULTS

It has previously been demonstrated that intravenous inoculation of rhesus monkeys with HFV-positive stool extracts of sporadic non-A, non-B hepatitis patients or experimentally infected monkeys caused an elevation of serum aminotransferases, and the infectious agent was excreted in the stools (9). HFV-positive stool specimens collected from one of such experimentally infected monkeys (MMU-46) was examined under an electron microscope. Spherical VLPs of various sizes were seen (data not shown). The small particles were in the range of 27 to 37 nm, and the large particles were 50 to 100 nm in size.

For separation of the large and small VLPs, the stool extract was centrifuged and the pellet was fractionated on a discontinuous sucrose gradient. Figure 1 shows the distribution of HFV Ag along the gradient as detected by ELISA. Three peaks of antigen activity were observed. Fractions from different peaks were individually pooled and examined under an electron microscope. Peak A consisted of large particles 50 to 100 nm in diameter. Peak B had mostly small particles 27 to 37 nm in diameter (Fig. 2), although occasionally large particles were also seen. Peak C had no particles but probably had soluble antigen or products of virus breakdown.

The two preparations of VLPs were injected into different rhesus monkeys to examine their infectivities. Monkeys MMU-36 and -37 were inoculated intravenously with small VLPs, while MMU-38 and -39 received the preparation containing large VLPs. The positive reference monkey, MMU-30, was given the stool extract that was used to purify the different-size particles. Antigen discharge and transaminase levels of all five monkeys were recorded. In MMU-30, a rise in SGPT and SGOT was observed on day 28 postinfection (p.i.) and persisted to day 59 (Fig. 3a). Antigen discharge started on day 18, peaked on day 21, and was not titratable after day 25 p.i. (Fig. 3a). In MMU-36 and MMU-37 also, the levels of SGPT and SGOT were elevated between days 28 and 59 p.i. (Fig. 3b and c). However, the peak levels of transaminases in both of these monkeys were much lower (about 50 to 70%) than those found in MMU-30. In these two monkeys, the period of antigen discharge was also shorter than in MMU-30, being only 4 days (between days 21 and 24 p.i.). Antigen-positive stool samples from MMU-30, -36, and -37 had both the large and small VLPs, as seen under an electron microscope. MMU-38 and MMU-39, inoculated with large particles, did not show any rise in serum transaminase levels until 70 days p.i. No HFV Ag was detectable in stool samples until 45 days p.i. (Fig. 3d and e).

These five monkeys, inoculated with either large or small VLPs or a mixture of the two, were challenged 90 days after the first inoculation with HFV Ag-positive stool extract (collected from MMU-36 on day 22 p.i.) which consisted of both the large and the small VLPs (Fig. 3f and g). While MMU-30, -36, and -37 showed no elevation of transaminase levels and no antigen discharge, which are characteristics of the disease, MMU-38 and -39, inoculated earlier with large VLPs, had antigen discharge from days 20 to 24 and elevation of transaminase levels between days 28 and 63.

Viral DNAs from 27- to 37-nm-size infectious fractions from stool and liver were resolved on an agarose gel and probed with ^{32}P-labeled viral DNA from stool (Fig. 4A). A 20-kb band was observed in the infected stool and liver (Fig. 4A, lanes S and L, respectively). The uninfected monkey liver (Fig. 4A, lane N) did not show the 20-kb HFV DNA. The 20-kb HFV DNA is double stranded, since it can be digested by DNase I in the presence of Mg^{2+} and by restriction enzymes. Cloning and sequencing of the entire 20-kb HFV DNA are currently under way. The presence of HFV DNA in infected liver establishes a correlation between infectivity of the virus and the purified viral preparation from stool. HFV has been successfully grown in human larynx carcinoma (HEp-2) cells (3a). Viral DNA isolated from the infected cells was found to be 20 kb in size, and preliminary restriction mapping indicated this DNA species to be the same as the viral DNA of infected monkey stool. DNA samples from uninfected and infected monkey and patient stool samples were probed with the HFV DNA isolated

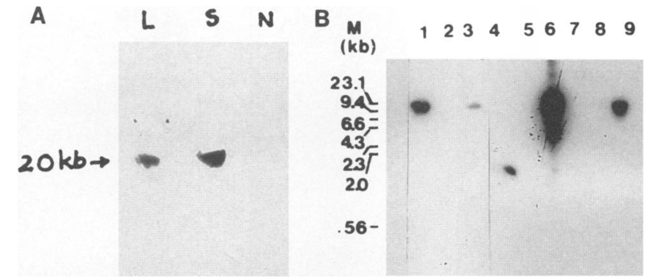

FIG. 4. Agarose gel electrophoresis of viral DNA isolated from liver (lane L) and stool (lane S) samples from infected monkeys and from a liver sample from an uninfected monkey (lane N). The filter was probed with the 27- to 37-nm VLP DNA (peak B in Fig. 1). (B) Agarose gel electrophoresis of viral DNA isolated from stool samples from uninfected and infected monkeys and a patient. Lanes: M, *Hin*dIII-digested λ marker. 1 to 3, sporadic non-A, non-B enteric hepatitis patient stool; 4 and 5, uninfected human stool; 6, cell culture viral DNA (HFV); 7, uninfected monkey stool; 8, empty lane; 9, infected monkey stool DNA. The filter was probed with the cell culture HFV DNA.

from cell culture (Fig. 4B). Two of three patient stools (lanes 1 and 3) and monkey stool (lane 9) exhibited a 20-kb band corresponding to HFV. Uninfected monkey (lane 7) and human (lanes 4 and 5) stools and one non-A, non-B enteric hepatitis patient stool (lane 2) did not exhibit the 20-kb viral DNA. Lane 6 contains the HFV DNA from cell culture. This result further proves that HFV can be detected among sporadic non-A, non-B enteric hepatitis patients and in the stools of monkeys infected with the virus. Uninfected monkey stool does not exhibit HFV DNA, thus ruling out the possibility that this virus originates as a passenger virus from the intestines of the monkeys. In addition, the 20-kb HFV DNA is detectable in 60% of non-A, non-B hepatitis patient stool samples from two cities Lucknow and Allahabad, in northern India (3b). ELISA screening of 222 coded serum and stool samples also confirms the prevalence rate of 60% HFV among non-A, non-B hepatitis patients. These results establish that HFV is associated with non-A, non-B hepatitis.

To establish if liver dysfunction as indicated by the elevation of serum aminotransferase levels was accompanied by any ultrastructural changes characteristic of hepatitis, liver biopsies were performed in experimentally infected monkeys. Characteristic hepatic lesions were observed in liver biopsy specimens from experimentally infected monkeys. Parenchymal degenerative changes varying in degree and fatty infiltration of the hepatocytes were noticed. Necrotic foci associated with infiltration of lymphomononuclear cells and polymorphs were also observed (Fig. 5a). In general, the sinusoidal space was moderately widened, with proliferation of Kupffer cells and mild sinusoidal lymphocytosis observed. Perivascular and periductular leukocytic infiltration in the portal areas (Fig. 5b) and proliferation of biliary ductules were also noticed. More advanced hepatopathic changes such as parenchymal necrotic areas associated with either focal and discrete or diffuse leukocytic infiltration of predominately lymphomononuclear cells and fibrocytes were noticed (Fig. 5c). The hepatic capsule was thickened moderately as a result of leukocytic infiltration, which at places extended deep into the parenchyma. Intracytoplasmic round or oval bodies were observed in the hepatocytes of one of the monkeys (Fig. 5d). By Lendrum's method of staining, these intracytoplasmic bodies appeared as bright red inclusions.

Ultrastructural observations revealed the presence of spherical VLPs in the cytoplasm of hepatocytes of one of the

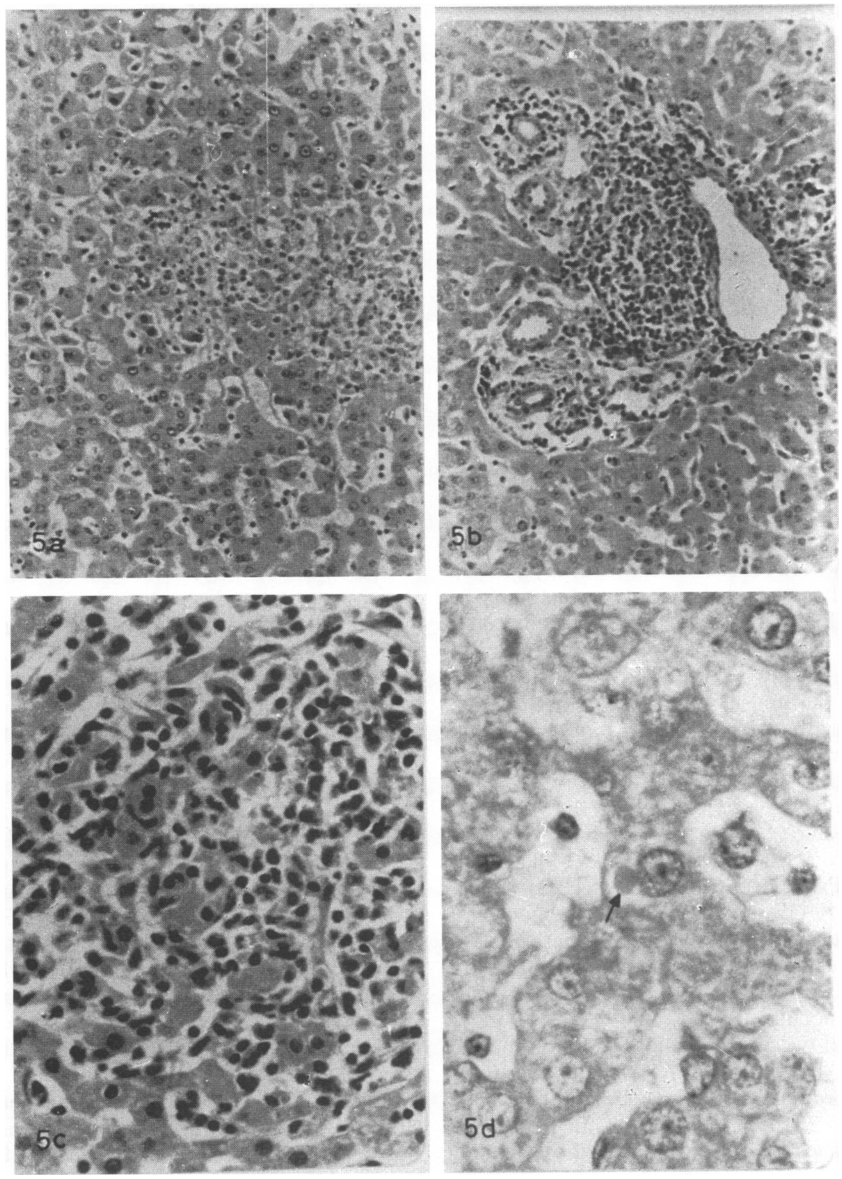

FIG. 5. Histopathological changes in liver of experimentally infected monkeys. (a) Necrotic foci associated with infiltration of lymphomononuclear and polymorphs (magnification, ×200); (b) perivascular and periductular leukocyte infiltration in the portal area (magnification, ×200); (c) leukocyte infiltration of fibrocytes (magnification, ×500); (d) intracytoplasmic body (arrow) (magnification, ×500).

experimentally infected monkeys (13). The sizes of these particles ranged between 30 and 80 nm.

DISCUSSION

Our experiments demonstrate that monkeys infected with sporadic non-A, non-B hepatitis patient stool extracts showed the presence of 27- to 37-nm and 50- to 100-nm VLPs. These were separated and used for infection of monkeys. The data show that 27- to 37-nm particles cause non-A, non-B hepatitis in monkeys, whereas 50- to 100-nm particles fail to induce the disease. Also, monkeys that had previously been inoculated with the small particles were protected when challenged de novo with the infectious material, whereas those inoculated with the large particles did not resist the challenge. These observations indicate that the 27- to 37-nm VLPs are responsible for non-A, non-B hepatitis and that infection with these particles confers immunity to animals against infection.

Liver biopsies taken from the infected monkeys during the acute phase of illness showed characteristic histopathological changes. The presence of intracytoplasmic oval inclusions was suggestive of a viral infection of the hepatocytes (8). Particles of both 27 to 37 nm and 60 to 80 nm were seen in the cytoplasm of hepatocytes of one of the experimentally infected monkeys (13). The presence of the 27- to 37-nm particles in hepatocytes is consistent with the finding that these particles are responsible for hepatitis. However, the role of the large particles is not clear. These particles are immunoreactive in a sandwich ELISA that detects the small VLPs. The large particles do not, however, appear to be involved in protective immunity.

The viral nature of the novel agent described here is further demonstrated by the fact that the same ~20-kb DNA moiety is amplified in cell culture (unpublished observation). Using the HFV DNA from cell culture as a probe, we further demonstrate that the virus was originally present in the stools of French patients and does not appear as a passenger virus from the monkey intestine during transmission.

Our studies indicate that 27- to 37-nm VLPs are the causative agent of non-A, non-B hepatitis in rhesus monkeys. HEV-positive patients shed HEV particles 27 to 34 nm in size. However, HEV is 7.5-kb-long RNA virus.

The presence of HEV RNA is detectable in stool by PCR (9). We screened stool samples from sporadic hepatitis patients for the presence of HEV and HFV and found that 40% (4 of 10) of the samples were HEV positive and 60% (6 of 10) were HFV positive (3b). In addition, an HFV Ag ELISA on 222 coded specimens also revealed the prevalence of this virus to be around 60%. The presence of the HFV genome in stool samples from non-A, non-B hepatitis patients and in liver and stool samples from infected monkeys further indicates that HFV is associated with non-A, non-B hepatitis.

The genetic material of HFV is a 20-kb long double-stranded virus. A 5-kb-long DNA non-A, non-B hepatitis virus has been sequenced (11). However, the size and preliminary restriction map of HFV does not resemble those of the 5-kb-long DNA virus or other enterically transmitted viruses. Sequencing of the entire viral genome may shed light on the nature of coding proteins.

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